Environmental Health Perspectives
Vol. 88, pp. 71–76, 1990

Rat Liver Endothelial and Kupffer Cell-Mediated Mutagenicity of Polycyclic Aromatic Hydrocarbons and Aflatoxin B_1

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The ability of isolated rat liver endothelial and Kupffer cells to activate benzo(*a*)pyrene (BP), *trans*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene (DDBP), *trans*-1,2-dihydroxy-1,2-dihydrochrysene (DDCH), and aflatoxin B_1 (AFB_1) to mutagenic metabolites was assessed by means of a cell-mediated bacterial mutagenicity assay and compared with the ability of parenchymal cells to activate these compounds. Endothelial and Kupffer cells from untreated rats were able to activate AFB_1 and DDBP; DDBP was activated even in the absence of an NADPH-generating system. Pretreating the animals with Aroclor 1254 strongly enhanced the mutagenicity of the dihydrodiol, whereas the mutagenicity of AFB_1 showed a slight increase. BP and DDCH were only activated by endothelial and Kupffer cells isolated from Aroclor 1254-pretreated rats. Parenchymal cells from untreated animals activated all four carcinogens tested; Aroclor 1254 enhanced the parenchymal cell-mediated mutagenicity of BP and DDCH but did not affect that of DDBP and clearly reduced that of AFB_1. The reduced mutagenicity of AFB_1 correlates with the decrease in the amount of 2α-hydroxytestosterone formed when testosterone was incubated with parenchymal cell microsomes from Aroclor 1254-pretreated rats (compared with microsomes from untreated animals): the formation of 2α-hydroxytestosterone is specifically catalyzed by cytochrome P-450*h*, a hemoprotein thought to be involved in the activation of AFB_1. These results show that not only rat liver parenchymal cells, but also endothelial and Kupffer cells, activate several carcinogens to mutagenic metabolites.

Introduction

In the mammalian liver, parenchymal cells account for more than 90% of the mass and 65% of the total number of cells. The nonparenchymal cells of the liver are primarily endothelial and Kupffer cells, which occupy the sinusoidal lining of the intact liver. All three of these cell types are heterogeneous in the way they respond to insult from xenobiotics. The basis of the heterogeneous responses to hepatotoxins is poorly understood, but several factors, including the distribution of the xenobiotics within the liver, differences in the DNA repair mechanisms among the cell populations, and differences in the ability to activate and detoxify these compounds, might be involved. It is the cell's ability to activate and detoxify hepatotoxins that we wish to address in the present study.

Recent studies have shown that in the sinusoidal lining cells oxidative enzyme activities (aminopyrine *N*-demethylase and ethoxyresorufin *O*-deethylase) are low, while postoxidative enzyme activities (glutathione *S*-transferase and epoxide hydrolase) are relatively high (*1,2*). The aim of this study was to analyze the capacity of sinusoidal lining cells, with their low cytochrome P-450-dependent enzyme activities, to activate several carcinogenic compounds to mutagenic metabolites and compare this capacity with that of parenchymal cells. The activating potential of the isolated liver cell subpopulations was estimated by means of a cell-mediated mutagenicity assay (*3*). To characterize the cytochrome P-450 profile of rat liver parenchymal cells, as well as nonparenchymal cells, from untreated and Aroclor 1254-pretreated rats, the oxidative metabolism of testosterone with microsomes from parenchymal, endothelial, and Kupffer cells was investigated. It has been shown recently that various forms of rat liver microsomal cytochrome P-450 catalyze the hydroxylation of testosterone with a high degree of regio- and stereoselectivity (*4,5*).

Materials and Methods

Male Sprague-Dawley rats (200–240 g body weight) were used. Aroclor 1254 in corn oil was administered as a single IP dose (500 mg/kg body weight) 5 days before killing. Control rats received appropriate volumes of corn oil (2.5 mL/kg body weight).

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Parenchymal, endothelial, and Kupffer cells were isolated and characterized according to methods previously described in the literature (*1*). For each mutagenicity assay, pools of Kupffer cells and of endothelial cells isolated from three untreated or Aroclor 1254-pretreated rats were prepared. All the experiments were performed in cell homogenates obtained by sonicating the cells for 30 sec at 60% duty cycle on a Branson cell disruptor (model B-15).

Reversion of *Salmonella typhimurium* his^-, a system developed by Ames et al. (*6*), was used for the estimation of mutagenicity. The incubation mixture consisted of 1 mL complete Krebs-Henseleit buffer containing previously sonicated cells (1×10^6 parenchymal cells or 10×10^6 nonparenchymal cells), bacteria (1.7×10^8 cells), and the test compounds dissolved in 10 μL dimethyl formamide. Incubations were supplemented with 2.5 mM $NADP^+$ and 2.0 mM glucose-6-phosphate. Samples were incubated in a shaking water bath at 37°C in the dark for 2 hr. Two milliliters of 45°C warm top agar, which contained 0.55% w/v agar, 0.55% w/v NaCl, 50 μM histidine, and 50 μM biotin in 25 mM sodium phosphate buffer (pH 7.4), was then added, and the mixture was poured onto a Petri dish containing 22 mL of minimal agar (1.5% agar in Vogel-Bonner E medium with 2% glucose). After incubation at 37°C in the dark for 3 days, colonies of his^+ revertants were counted.

Testosterone hydroxylation assays were performed by incubating microsomes (1 mg protein) from parenchymal, endothelial, and Kupffer cells for 30 min with 1 mM testosterone in the presence of 0.6 mM $NADP^+$, 8 mM glucose-6-phosphate, 1.4 units of glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$. Testosterone metabolites were extracted with dichloromethane and quantified by HPLC as previously described (*7*). Endothelial and Kupffer cells from three animals were pooled for the preparation of microsomes.

Results

Characterization of Isolated Cell Populations

The total yield of isolated parenchymal cells after collagenase digestion of the liver was $372 \pm 45 \times 10^6$ cells/rat liver ($n = 4$), which included 3 ± 1% endothelial cells, 4 ± 1% Kupffer cells, and 3 ± 2% fat-storing cells. The endothelial cell fractions ($37 \pm 5 \times 10^6$ cells/rat liver, $n = 24$) were contaminated with 9 ± 2% lymphocytes and 4 ± 2% Kupffer cells. The Kupffer cell preparations ($51 \pm 7 \times 10^6$ cells/rat liver, $n = 24$) were found to contain 10 ± 3% endothelial cells, 2 ± 2% fat-storing cells, and 0.2 ± 0.1% parenchymal cells.

Pretreatment of the animals with Aroclor 1254 (a polychlorinated biphenyl mixture that exhibits both phenobarbital and 3-methylcholanthrene-inducing properties) did not affect the yield, viability, and purity of the isolated parenchymal, endothelial, and Kupffer cell fractions, but it significantly increased the protein concentrations of parenchymal and nonparenchymal cells by about 80 and 30%, respectively.

Mutagenicity Studies

The cell-mediated mutagenicity assays were performed with roughly equal protein concentrations of parenchymal and nonparenchymal cells, i.e., 1×10^6 parenchymal cells and 10×10^6 nonparenchymal cells; only in the case of *trans*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene (DDBP), 0.5×10^6 parenchymal cells and 5×10^6 nonparenchymal cells were used. None of the compounds investigated was mutagenic in the absence of cell homogenates.

Parenchymal cells isolated from untreated animals showed a limited capacity to activate benzo(*a*)pyrene (BP) (Fig. 1*A*), whereas endothelial and Kupffer cells were unable to activate this compound (Fig. 1*B*); this was still the case if the cell number and/or the incubation time were doubled (data not shown). After administration of Aroclor 1254, a clear mutagenic effect of BP, mediated by parenchymal as well as nonparenchymal cells, was observed (Figs. 1*A* and *B*).

DDBP was activated by parenchymal, endothelial, and Kupffer cells isolated from untreated rats (Figs. 1*C* and *D*). Interestingly, this also occurred in all three cell types in the absence of the NADPH-generating system. Pretreatment of the animals with Aroclor 1254 enhanced the nonparenchymal cell-mediated mutagenicity of the dihydrodiol, while it did not alter the mutagenic effect mediated by the parenchymal cells (Figs. 1*C* and *D*).

Parenchymal cells, but neither endothelial nor Kupffer cells, from untreated rats were able to activate *trans*-1,2-dihydroxy-1,2-dihydrochrysene (DDCH) to a mutagenic metabolite (Figs. 1*E* and *F*). After administration of Aroclor 1254, all three cell types were very effective in activating DDCH (Figs. 1*E* and *F*).

Aflatoxin B_1 (AFB_1) was strongly mutagenic after incubation with parenchymal and nonparenchymal cells isolated from untreated animals (Figs. 1*G* and *H*). Pretreatment of the rats with Aroclor 1254 slightly enhanced the mutagenicity of the mycotoxin mediated by endothelial and Kupffer cells, whereas it significantly decreased the activating potential of the parenchymal cells (Figs. 1*G* and *H*).

Testosterone Metabolism

The oxidation products identified after incubating testosterone with parenchymal cell microsomes from untreated rats were 6α-, 7α-, 6β-, 16α-, 16β-, 2α-, and 2β-hydroxytestosterone and androstenedione (Fig. 2). The hydroxylation of testosterone occurred at a significantly lower rate in microsomes of endothelial and Kupffer cells; furthermore, neither 16β- nor 2β-hydroxytestosterone could be detected with these microsomes. If incubations were performed with microsomes of parenchymal and nonparenchymal cells from Aroclor 1254-pretreated rats, the formation rate of all the testoster-

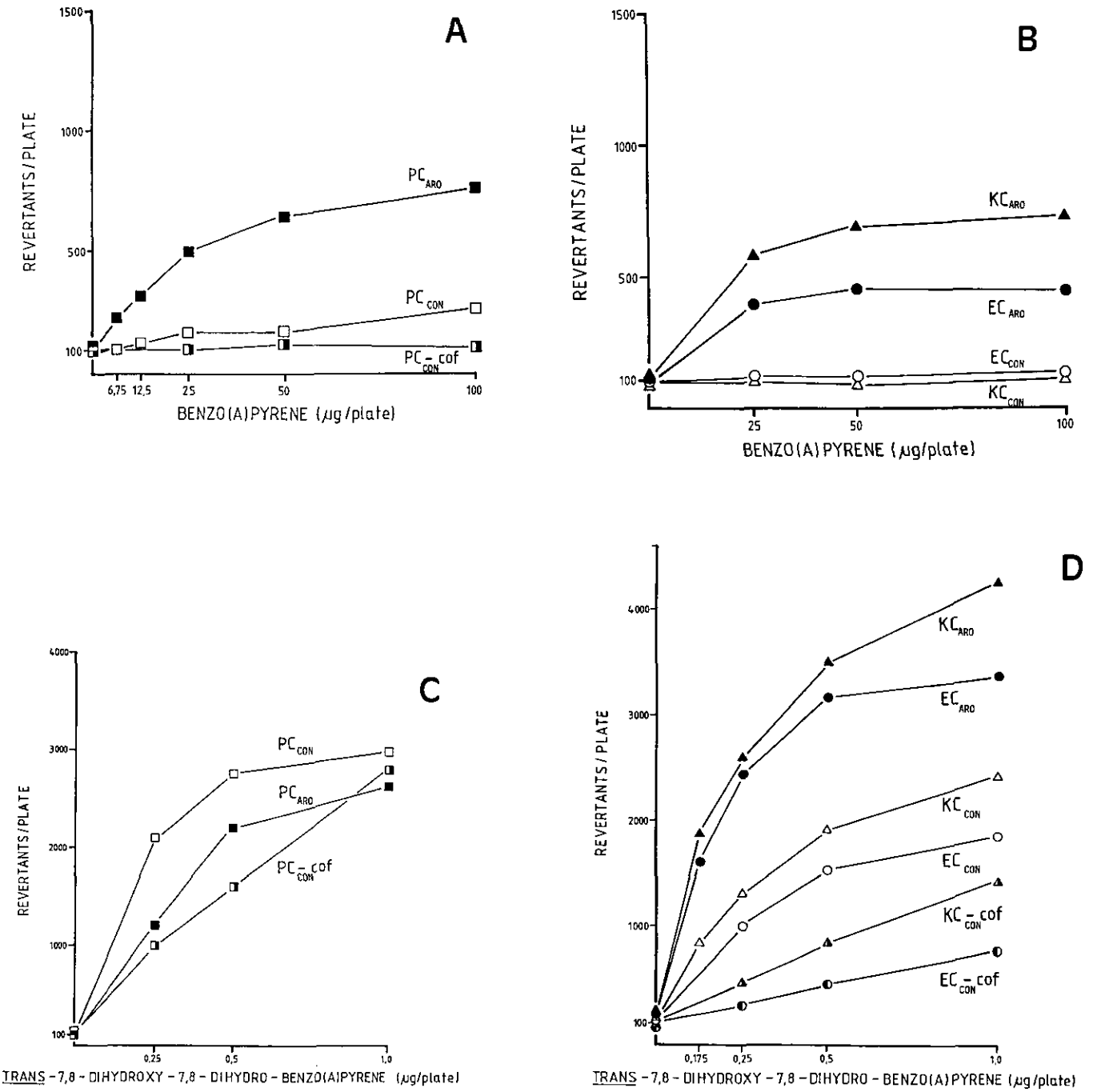

FIGURE 1. Parenchymal and nonparenchymal cell-mediated mutagenicity of three polycyclic aromatic hydrocarbons and aflatoxin B_1. Homogenates of parenchymal (PC), endothelial (EC), and Kupffer cells (KC) isolated from untreated (CON) or Aroclor 1254-pretreated (ARO) rats were incubated with *S. typhimurium* TA 100 or TA 98 (in the case of aflatoxin B_1) and the indicated amount of benzo(*a*)pyrene (*A,B*); *trans*-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene (*C,D*); *trans*-1,2-dihydroxy-1,2-dihydrochrysene (*E,F*), or aflatoxin B_1 (*G,H*). The assays with cells from untreated animals were carried out in the presence or absence (-cof) of a NADPH-generating system, whereas all those with cells from induced animals were carried out in the presence of $NADP^+$ and glucose-6-phosphate. Values are means of two experiments, each of them comprising duplicate incubations. For each experiment, nonparenchymal cells from three rats were pooled. (*Continued on next page.*)

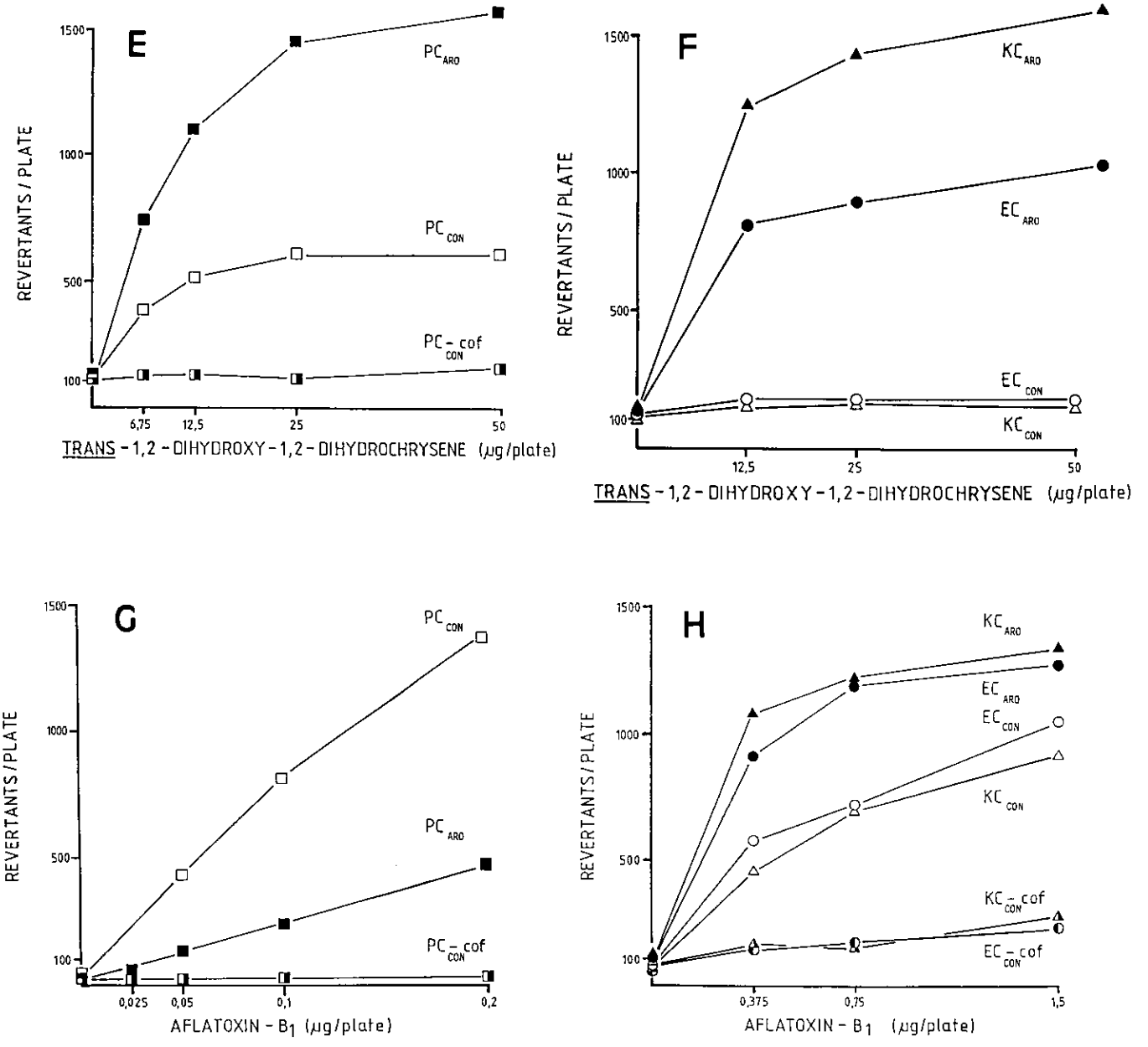

FIGURE 1. *Continued.*

one metabolites increased, with one exception: parenchymal cell microsomes formed 2α-hydroxytestosterone at about one-third of the rate observed in controls, while the formation rate of this metabolite in nonparenchymal cells remained unchanged.

Discussion

Bay-region diol epoxides are now well established as principal ultimate carcinogenic metabolites of the polycyclic aromatic hydrocarbon class of carcinogens. Three metabolic steps are involved in the conversion of a polycyclic aromatic hydrocarbon into its bay-region diol epoxides: formation of an arene oxide, which is catalyzed by cytochrome P-450; hydrolysis of the arene oxide to the *trans*-dihydrodiol, a step that requires epoxide hydrolase; a second oxygenation by cytochrome P-450 to yield the diol epoxide. In parenchymal, endothelial, and Kupffer cells, no significant activation of BP was observed, a fact that can be attributed to the very low levels of cytochrome P-450 forms catalyzing the metabolic steps in these cells (*8*). After administration of Aroclor 1254, BP was activated by all three cell types, this effect being accompanied by a strong increase in the cytochrome P-450 content of parenchymal and nonparenchymal cells (*8*).

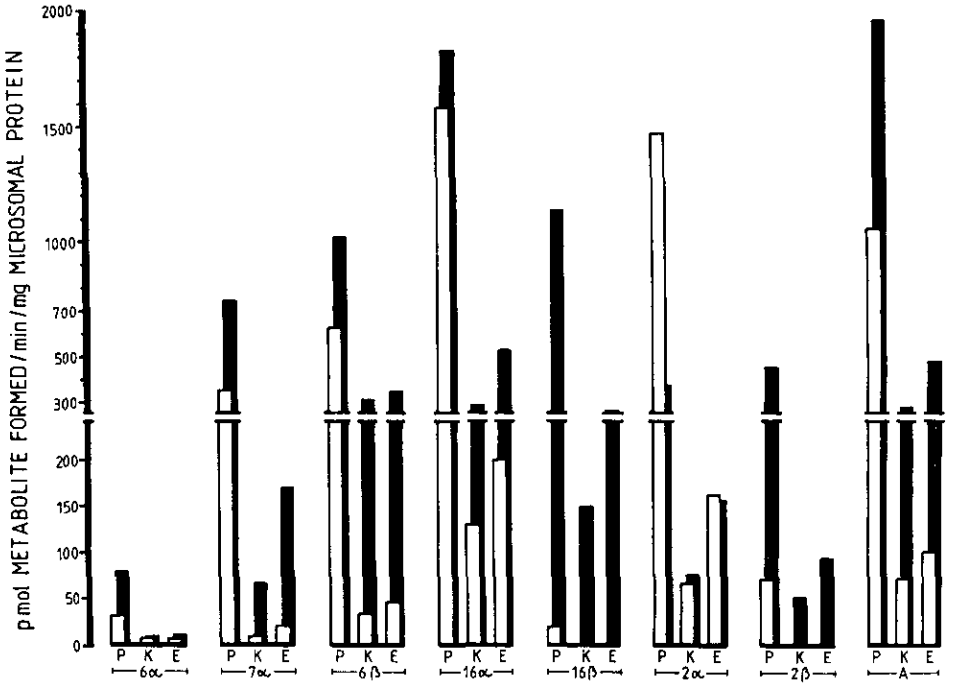

FIGURE 2. Testosterone hydroxylation by microsomes of parenchymal and nonparenchymal cells. Microsomes of parenchymal (P), endothelial (E), and Kupffer cells (K) isolated from untreated (open bars) or Aroclor 1254-pretreated rats (solid bars) were incubated for 30 min with 1 mM testosterone; the metabolites were extracted with dichloromethane and quantified by HPLC. The products identified were 6α-, 7α-, 6β-, 16α-, 16β-, 2α-, and 2β-hydroxytestosterone as well as androstenedione. The bars represent the means of two experiments; for each experiment, endothelial and Kupffer cells from three animals were pooled.

The striking ability of parenchymal, endothelial, and Kupffer cells from untreated rats to activate DDBP so effectively (compared to BP) may be due to the fact that the dihydrodiol is the immediate precursor of the highly mutagenic diol epoxide, the amount of cytochrome P-450 present in untreated cells being sufficient to activate this compound with a high degree of efficiency. Alternatively, the conversion of the dihydrodiol to the diol epoxide can be catalyzed by prostaglandin endoperoxide synthetase during the oxidation of arachidonic acid by prostaglandins (*9,10*). Present studies are aimed at elucidating the possible role of prostaglandin endoperoxide synthetase in the activation of dihydrodiols derived from polycyclic aromatic hydrocarbons by isolated rat liver cells.

DDCH was activated by parenchymal cells from untreated and Aroclor 1254-pretreated rats, while only endothelial and Kupffer cells from induced animals were able to activate the dihydrodiol. DDCH is a rather poor substrate for the cytochrome P-450 monooxygenase system: it is metabolized by liver microsomes from 3-methylcholanthrene-pretreated rats at about 8% of the rate at which DDBP is metabolized (*11*). Thus, the observation that endothelial and Kupffer cells from untreated animals were unable to activate DDCH might again be ascribed to the very low amount of cytochrome P-450 in these cells.

AFB_1 was activated by parenchymal and nonparenchymal cells isolated from untreated rats; this finding suggests that a constitutive form(s) of cytochrome P-450 is involved in the activation of the mycotoxin. In accordance with this proposal, it has recently been shown that four cytochrome P-450 isoenzymes purified from untreated animals were able to activate AFB_1 in a reconstituted monooxygenase system (*12*); among them was cytochrome P-450*h*, a male-specific form of cytochrome P-450. Pretreatment of the rats with Aroclor 1254 significantly decreased the parenchymal cell-mediated mutagenicity of AFB_1, whereas the mutagenic potential of the hepatotoxin in the presence of induced endothelial and Kupffer cells was slightly enhanced when compared to control cells. Furthermore, the formation rate of 2α-hydroxytestosterone, which reflects the levels of cytochrome P-450*h*, was strongly reduced in parenchymal cells of induced rats, while it remained unchanged in nonparenchymal cells. Thus, cytochrome P-450*h* seems to be involved in the activation of AFB_1.

However, as mentioned earlier, other constitutive cytochrome P-450 forms might also mediate the activation of the mycotoxin. Hepatic S-9 preparations from newborn male rats were able to do so (data not shown), although cytochrome P-450*h* was not detected in these livers. On the other hand, the strong reduction in the parenchymal cell-mediated mutagenicity of AFB_1 after administration of Aroclor 1254 might be due (at least in part) to the induction of AFB_1-4-hydroxylase (*13*). This enzyme catalyzes the conversion of AFB_1 to aflatoxin

M_1, the latter compound being with and without further metabolism less than 5% as active as AFB_1.

In the present study liver cells were homogenized and a NADPH-generating system was added to the incubation medium. The homogenization leads to the dilution of several cofactors (e.g., glutathione), whereas the addition of $NADP^+$ and glucose-6-phosphate fortifies the cytochrome P-450-dependent monooxygenase system. Taking into account that the major detoxification route of AFB_1-8,9-epoxide is its conjugation with glutathione, catalyzed by glutathione *S*-transferase (*14*), the result of mutagenicity assays might be different if whole cells are used. Present experiments are aimed at clarifying this issue.

In conclusion, this study shows that rat liver parenchymal as well as endothelial and Kupffer cells are able to activate several carcinogens to mutagenic metabolites. Interestingly, DDBP is activated by all three cell types even in the absence of an NADPH-generating system, while constitutive cytochrome P-450 forms seem to be involved in the activation of AFB_1.

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 302. We thank I. Böhm for typing this manuscript.

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